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FUM9 Is Required for C-5 Hydroxylation of Fumonisins and Complements the Meitotically Defined *Fum3* Locus in *Gibberella moniliformis*

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Deletion of the *Gibberella moniliformis FUM9* gene resulted in mutants that produce only fumonisins that lack a C-5 hydroxyl group. This phenotype is identical to that of previously described mutants with defective alleles at the meiotically defined *Fum3* locus. Transformation with a wild-type *FUM9* gene into a *Fum3*-defective mutant restored wild-type fumonisin production. These results indicate that the *FUM9* protein catalyzes the C-5 hydroxylation of fumonisins and that *FUM9* and the *Fum3* locus are the same gene.

Gibberella moniliformis is one of the fungi most commonly associated with maize worldwide. The fungus can cause severe ear and stalk rots, but also is present in healthy maize tissue (9) and can produce high concentrations of the carcinogenic fumonisin mycotoxins. In naturally infected maize, wild-type strains of the fungus produce fumonisins B_1 (FB_1), B_2 (FB_2), B_3 (FB_3), and B_4 (FB_4) (13). These toxins are synthesized via polyketide metabolism and consist of a linear 20-carbon backbone with an amine, one to three hydroxy moieties, and two methyl and two tricarboxylate functions at various positions along the backbone.

Genetic analysis of naturally occurring variants (10) identified four loci required for fumonisin biosynthesis (5–7, 11): *Fum1* confers the ability to produce fumonisin, *Fum2* confers the ability to hydroxylate carbon atom 10 (C-10) of the fumonisin backbone, *Fum3* confers the ability to hydroxylate carbon atom 5 (C-5) of the backbone, and *Fum4* controls the amount of fumonisins produced. For example, strains with a defective *Fum3* allele produce only fumonisins that lack a C-5 hydroxyl (i.e., FB_3 and FB_4) (6, 15).

The fumonisin biosynthetic cluster in *G. moniliformis* contains 15 coregulated genes, designated *FUM1* and *FUM6* through *FUM19* (14). *FUM9* is one of seven genes in the *G. moniliformis* fumonisin biosynthetic gene cluster that has not been functionally characterized. Amino acid comparison of the predicted *FUM9* protein yielded only low levels of similarity to dioxygenases. However, the predicted *FUM9* protein does share significant homology to actinomycete sequences, which in turn are similar to oxoglutarate-dependent dioxygenases (18). Here, we report the results of deletion of *FUM9*, sequence analysis of a naturally occurring mutant with a fumonisin phenotype identical to the *FUM9* deletion mutant, and complementation of the naturally occurring mutation with the wild-type *FUM9* gene.

To study the role of *FUM9* in fumonisin biosynthesis, we first deleted the gene. A *FUM9* deletion vector, pFUM9KOH, was constructed as previously described (2, 3). Briefly, the 1-kb regions immediately upstream and downstream of the *FUM9* coding region were amplified by PCR and subcloned into the same vector so they were separated by an *Asc*I restriction site. The hygromycin B resistance gene (*HygB*) was then inserted between the two fragments, utilizing the *Asc*I site to yield vector pFUM9KOH. Primers 9-1, 9-2, 9-4, and 9-4 were used to amplify these regions (Fig. 1 [primer sequences: 9-1, GACG GATCCGCGGCCTATTGGGACGTACTA; 9-2, GACGGCGCGCCTGC ATTGGCGTTGGCAAA; 9-3, GACGGCGCGCCGACGTTTGAATTGT CTTGGCGT; 9-4, GACCTCGAGGGCAACAAACTCCCTGCAAT; 9-5, TCAAGTTCCTCGTAATCGC; 9-6, CACAAGTGGGAGTTCAACC; 9-7, GAAGGTGATGAAGTGTCGG; 10-1, GACACGCGTCAAGGAAATTGG CGCACATAG; rp250, CTGCTGCATTCCCATTCCCATCGT; 1098, ACCAAGCCTATGCCTACAGCATCC]). All PCR products were generated with *Pfu* polymerase and sequenced to confirm the absence of errors.

To delete *FUM9*, wild-type *G. moniliformis* strain M-3125 (8) was transformed with the *FUM9* deletion vector via the protoplast method, and transformants were selected on hygromycin B-amended regeneration medium as previously described (3, 16). Eighteen hygromycin B-resistant transformants were recovered and screened by PCR to identify those in which the *FUM9* coding region was deleted. The PCR strategy was designed to amplify unique combinations of sequence elements that were formed from homologous recombination between the deletion vector and the *FUM9* locus. The PCR primers used for this screen were 9-5, rp250, 1098, and 9-7 (Fig. 1). Transformants also were screened by PCR for the absence of the *FUM9* coding region by using primers 9-5, 9-6, 10-1, and 9-7 (Fig. 1). Selected transformants were regenerated from single conidia to ensure genetic homogeneity and then subjected to Southern analysis to confirm deletion of the *FUM9* coding region. The Southern analysis employed standard protocols (17) and probes labeled with ^{32}P by the RediPrime kit (Amersham Biosciences, Piscataway, N.J.). The PCR screens and Southern analysis identified two transformants, GMT-9-206 and GMT-9-211, in which the *FUM9* coding region was deleted and replaced with the *HygB* gene (Fig. 1).

Fumonisin production in transformants was assessed by liquid chromatography-mass spectroscopy of acetonitrile-water

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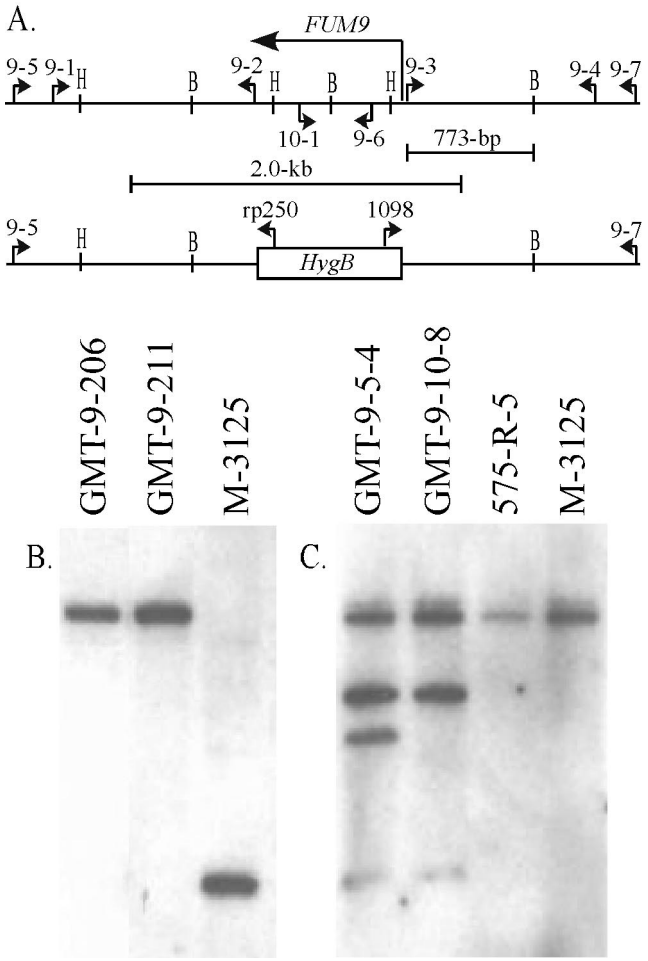

FIG. 1. Southern analysis of *FUM9* deletion mutants and 575-R-5 complemented with *FUM9*. (A) Genomic region of the wild-type (top) and the deleted (bottom) *FUM9* coding region (large arrow). H, *Hin*dIII; B, *Bgl*II. Small arrows indicate positions and orientations of PCR primers. (B) *Bgl*II-digested genomic DNA from *FUM9* deletion mutants probed with the 773-bp fragment shown in panel A. Note in the mutants GMT-9-206 and GMT-9-211 the loss of the wild-type 1.2-kb *FUM9* fragment and the gain of the 3.6-kb fragment resulting from integration of vector sequences by double-homologous recombination and the resulting replacement of the 0.9-kb *FUM9* coding region with *HygB*. (C) *Apa*I-digested genomic DNA from *Fum3*-complemented mutants, GMT-9-5-4 and GMT-9-10-8, hybridized to a 2.0-kb fragment carrying the entire *FUM9* coding region, including 453 bp upstream of the start site and 672 bp downstream of the stop site. Note additional hybridizing bands in DNA from complemented strains.

(1:1) extracts of 3-week-old cracked corn cultures as previously described (12, 16). The only fumonisins produced by the *FUM9* deletion mutants were FB_3 and FB_4. In contrast, the wild-type complement of FB_1, FB_2, FB_3, and FB_4 was produced by transformants in which *FUM9* remained intact. Because the C-5 hydroxyl is the only structural feature that is absent in both FB_3 and FB_4 but present in FB_1 and FB_2, these data indicate that *FUM9* is required for the C-5 hydroxylation of the fumonisin backbone.

The *FUM9* deletion mutants had the same phenotype as previously described mutants with defective alleles of the meiotically defined *Fum3* locus. To determine if *Fum3*-defective mutants carry mutations in *FUM9*, we amplified and sequenced a 1,591-bp fragment spanning the *FUM9* coding region from strain 575-R-5, which has the mutant *Fum3-3* allele and therefore cannot hydroxylate the C-5 position of fumonisins. 575-R-5 is a progeny from a sexual cross of wild-type strain M-3125 and a UV-induced mutant derived from wild-type strain M-3120 (15). The sequence of the *FUM9* coding region in strain 575-R-5 was identical to that of M-3125, except for a C-to-T transition at nucleotide 94 that is predicted to introduce a stop codon (ochre mutation) in the coding region and a G-to-C transversion at nucleotide 495 that is predicted to result in no amino acid change. Sequence analysis of the same region of DNA in M-3120, which produces the wild-type complement of FB_1, FB_2, FB_3, and FB_4 (6), showed that it also carries the G-to-C transversion.

TABLE 1. Fumonisin production by wild-type *G. moniliformis* strain M-3125, *Fum3*-defective mutant 575-R-5, and transformants GMT-9-5-4 and GMT-9-10-8

Strain[a]	% Fumonisin recovered		
	FB_1	FB_2	FB_3
M-3125	77	16	7
575-R-5	0	0	100
GMT-9-5-4	71	14	15
GMT-9-10-8	37	33	30

[a] Transformants GMT-9-5-4 and GMT-9-10-8 were generated by transforming 575-R-5 with a vector (pUCH2-8F9) carrying a wild-type copy of *FUM9*.

To determine if *FUM9* can complement a *Fum3*-defective mutant, we transformed 575-R-5 with a wild-type copy of *FUM9*. A 2.0-kb genomic region containing the wild-type *FUM9* coding region was amplified via PCR with *Pfu* DNA polymerase from cosmid clone 4-5 (14). Nucleotide sequence analysis indicated that the amplified *FUM9* did not have any errors. The 2.0-kb amplification product was subcloned into the hygromycin B-containing vector pUCH2-8 (1) to yield the complementation vector pUCH2-8F9. 575-R-5 was transformed with circular pUCH2-8F9, and hygromycin B-resistant putative transformants were recovered and demonstrated by PCR to carry the vector. Southern analysis of two selected transformants revealed that one, GMT-9-5-4, had multiple copies of pUCH2-8F9, while the second, GMT-9-10-8, had only one copy of the vector (Fig. 1). Fumonisin production assays, as described above, revealed that both GMT-9-5-4 and GMT-9-10-8 produced the wild-type complement of B-series fumonisins (Table 1). These results indicate that the wild-type copy of *FUM9* complemented the *Fum3*-defective mutant 575-R-5 and therefore provide further evidence that the ochre mutation in the *FUM9* coding region of this mutant results in its altered fumonisin production phenotype.

In this study, we have identified a direct link between the *FUM9* gene and hydroxylation of the C-5 position of the fumonisin backbone. *FUM9* deletion mutants produce only FB_3 and FB_4, which lack the C-5 hydroxyl, but not FB_1 and FB_2, which have the hydroxyl. These results suggest that the *FUM9* protein is a fumonisin C-5 hydroxylase. This conclusion is consistent with the prediction that *FUM9* encodes an oxoglutarate-dependent dioxygenase (18). Such dioxygenases frequently catalyze hydroxylation reactions (4).

Our results also reconcile classical and molecular genetic analyses of fumonisin biosynthesis in *G. moniliformis* by demonstrating that the meiotically defined *Fum3* locus and the molecularly defined *FUM9* are the same gene. The first evidence for this identity was that the *FUM9* deletion mutants had the same phenotype as *Fum3* mutants. Further evidence was that the *FUM9* coding region in *Fum3*-defective mutant 575-R-5 had an ochre mutation that should result in a truncated *FUM9* protein. The final evidence was that a wild-type copy of *FUM9* could complement the *Fum3*-defective mutant. Based on these results, we propose that hereafter *FUM9*/*Fum3* be designated *FUM3*. This designation is consistent with the conventional designation of fumonisin biosynthetic genes (i.e., *FUM*) and with the precedent set by *Fum3* being described before *FUM9*.

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Names are necessary to report factually on available data. However, the USDA neither guarantees nor warrants the standard of these products, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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